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Thanks,

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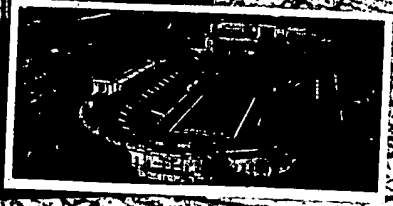
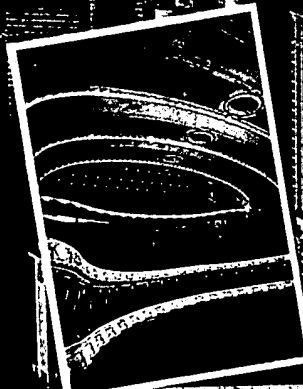
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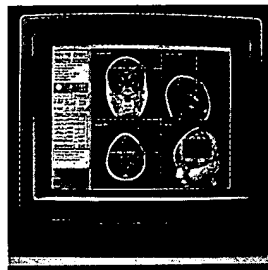
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Detection and Quantification of Vascular Endothelial Growth Factor/Vascular Permeability Factor in Brain Tumor Tissue and Cyst Fluid: The Key to Angiogenesis?

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IN PRIMARY MALIGNANT brain tumors increased vascularity and marked edema strongly suggest a possible role of the vascular endothelial growth factor/vascular permeability factor (VEGF/VPF). This was confirmed by earlier in situ hybridization studies, by analysis of the expression of the mitogen in different subsets of glioblastoma cells, and by the fact that the VEGF/VPF receptor *flt-1* (*fms*-like tyrosine kinase) is up-regulated in tumor cells in vivo. To assess and quantify the expression of the VEGF/VPF gene and of the receptor gene, 26 surgical specimens of brain tumor tissue from 24 patients were analyzed. In most malignant gliomas, the expression level of the VEGF/VPF gene is elevated and can be increased up to 20- to 50-fold in comparison with low-grade tumors. Using polymerase chain reaction-based amplification, it could be shown that the messenger RNAs of three different VEGF/VPF forms are synthesized in tumor tissue samples. Northern blot studies revealed that in some samples a significant expression of the gene coding for placenta growth factor, a growth factor closely related to VEGF/VPF, was observed. In addition, using a radioreceptor assay it was possible to detect high VEGF/VPF-like activity in the cyst fluids of brain tumors, indicating the accumulation of the mitogen and permeability factor in brain tumor cysts. Further investigations revealed that astrocytoma and glioblastoma cells in culture express the VEGF/VPF gene and secrete the VEGF/VPF protein, whereas gene expression of the two known VEGF/VPF receptors, kinase insert domain-containing receptor and *flt-1*, could not be detected. These data support previous reports, which stated that VEGF/VPF acts as a paracrine growth and permeability factor in brain tumors and may contribute to tumor growth by initiating tumor angiogenesis. (Neurosurgery 35:439-449, 1994)

Key words: Angiogenesis, Cyst fluid, Glioblastomas, Vascular endothelial growth factor, Vascular permeability factor

Vascular endothelial growth factor (VEGF) was discovered as a potent and specific endothelial cell mitogen in vitro (26, 28) and as a strong inducer of angiogenesis in vivo (41, 60). It may play a role in the development and maintenance of normal and tumor-associated vasculature (5, 10, 40, 44, 47, 52). VEGF is also known as vascular permeability factor (VPF) and is about 1000-fold more potent than histamine in inducing capillary permeability (11); it also can induce the release of von Willebrand-Factor (6) and thromboplasmin activity from human endothelial cells (9). This indicates that besides being angiogenic, VEGF/VPF also may enhance edema formation and may facilitate endovascular thrombosis (21).

VEGF/VPF is a preferentially secreted M_r 36,000 to 46,000 dimeric glycoprotein and has been identified from the conditioned medium of several cell lines (16, 17, 22). Molecular cloning of the complementary DNAs (cDNAs) has revealed four species of VEGF/VPF in human cells so far. These forms are generated by alternative splicing of the VEGF/VPF gene (24, 57) and they are designated VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆. The most abundant and best characterized form is VEGF₁₆₅, which is a secretable and heparin-binding isoform (18). A growth factor closely related to VEGF/VPF is the recently identified placenta growth factor (PlGF), which was found in human placenta (30). PlGF, like VEGF/VPF, is a secretory dimeric glycoprotein, and the PlGF protein shows a

similarity of approximately 50% to human VEGF/VPF. Like VEGF/VPF, PIGF also stimulates the growth of vascular endothelial cells, occurs as different forms (23, 31), and competes with [125 I]VEGF₁₆₅ for receptor binding (23).

High-affinity binding sites for VEGF/VPF were found on the surface of several kinds of vascular endothelial cells (4, 41, 42) and on various endothelial cell lines (38). Colocalization of [125 I]VEGF/VPF binding with factor VIII-like immunoreactivity proved the existence of VEGF binding sites on vascular endothelial cells (25). Recently, a receptor-type tyrosine kinase encoded by the *flt-1* gene (*fms*-like tyrosine kinase) was shown to bind VEGF/VPF with high affinity (15). The messenger RNA (mRNA) of *flt-1* is expressed in a variety of normal tissues of human and rat with high expression levels in human placenta, but not in most of the tumor cell lines so far examined (50). A second receptor tyrosine kinase was cloned from human endothelial cells and designated kinase insert domain-containing receptor (KDR) (55). Very recent results indicate that KDR encodes a second receptor for VEGF/VPF (36, 56), and that both receptors are expressed in human microvessel and large-vessel endothelial cells (2).

Malignant brain tumors show increased vascularity and are usually embedded in edematous brain tissue. Vascular permeability activity was demonstrated earlier in connection with many tumor studies, and it was also identified in the conditioned media of brain tumor cells (12, 13, 48, 49). More recent studies showed that capillary permeability-enhancing activity is not only present in the conditioned media of glioblastoma multiforme cells but also can be detected in brain cyst fluids, in which the activity can be blocked by neutralizing antibodies against VEGF/VPF (3). These studies also showed that there is a significant elevation of VEGF/VPF gene expression in highly vascularized and edema-associated brain tumors if compared with brain tumors with less neovascularization and little or no edema. In earlier studies we were able to show that the expression of the VEGF/VPF gene is induced in astrocytoma cells but is dramatically up-regulated in two apparently different subsets of glioblastoma cells *in vivo* (40). The VEGF/VPF protein seems to accumulate in the capillaries of tumor vessels. Furthermore, one of the high-affinity tyrosine kinase receptors for VEGF/VPF, *flt-1*, was not found in the endothelium of normal brain vessels, but the expression level was up-regulated in tumor endothelial cells *in vivo* (40). Recently Goldman and collaborators (21) were also able to demonstrate that VEGF/VPF is present in human glioma cell lines and in human glioblastoma multiforme surgical specimens. Stimulation of the epidermal growth factor receptor in those cell lines leads to an increase of the secretion of bioactive VEGF/VPF by 25% to 125% (21). Very recent results demonstrated that blocking of VEGF/VPF activity secreted from tumor cells in nude mice has a significant inhibitory effect on tumor growth (27). These observations strongly support the concept that tumor angiogenesis is regulated by paracrine mechanisms and identify VEGF/VPF as a potential tumor angiogenesis factor *in vivo*. They also provide a first link in our understanding about the cellular pathophysiology of glioma-associated tumor angiogenesis, increased vascular permeability, and cellular proliferation. The present study was undertaken to verify the

synthesis of VEGF/VPF in human brain tumors of central neuroepithelial origin and the expression of the VEGF/VPF receptor genes. A further aim was to assess the concentration of VEGF/VPF in fluid samples from brain tumor cysts.

MATERIALS AND METHODS

Cell lines

Fetal bovine aortic endothelial cells and human umbilical vein endothelial cells were maintained at 37°C as previously described (17, 58). The human glioblastoma cell line HGBM-1 (35) was a gift from Dr. Joseph Megyesi (Harvard Medical School & Children's Hospital, Department of Surgery, Boston, MA). The human astrocytoma cell line SNB-19, the human glioblastoma cells, and the retinoblastoma cell line Y79 were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. RNA from human omental microvascular endothelial cells was a gift from Dr. Michihiko Kuwano (Oita Medical University, Department of Biochemistry, Japan).

Tissue specimens and tumor cyst fluids

Twenty-six specimens of tumor tissue were obtained during 25 surgical procedures on 24 consecutive brain tumors. Meticulous efforts were made to obtain tissues from the very tumor core exclusively and to avoid an admixture with transitional or adjacent gliotic brain tissue. Specimens were frozen and immediately stored at -70°C. Histological typing was established according to current principles of neuropathology (7, 45, 46), and grading was based on the World Health Organization classification of brain tumors (61). The tumors were identified as pilocytic astrocytoma (one case), low-grade astrocytoma (three cases), anaplastic astrocytoma (two cases but three surgical procedures), glioblastoma (six cases but seven surgical procedures), anaplastic oligoastrocytoma (one case), oligodendroglioma (one case), and medulloblastoma (two cases). Additionally tissues from six meningiomas, one adenocarcinoma metastasis, and one anaplastic not classified tumor were used for analysis. Fluid samples with volumes varying between 10 and 30 ml were obtained at different times from four anaplastic astrocytomas (10 samples) during stereotactic biopsy procedures and by percutaneous tapping via an implanted catheter connected to a subcutaneous reservoir. The samples were kept at -70°C. Immediately before assay, the fluids were thawed and centrifuged, and the supernatant was filtered.

Preparation of probes

The VEGF/VPF cDNA was generated as described before (59), and the PIGF cDNA fragment generated from human placenta was used previously (23). The *flt-1* probe was a 1.1-kilobase human cDNA fragment (50), generated by polymerase chain reaction (PCR) (2), and the human KDR probe was a 1.4-kilobase cDNA fragment isolated as described before (56).

RNA isolation, Northern blot, and densitometric analysis

Total RNA was isolated by the guanidinium cesium chloride centrifugation method (8), subjected to electrophoresis on a 1.25% agarose gel containing 2.2 mol/L formaldehyde according to the surface-tension method, and transferred to a nitrocellulose membrane by capillary blotting. Blots were prehybridized and hybridized at 65°C as described before (59). The membranes were finally washed with 0.1 × standard saline citrate containing 0.1% sodium dodecyl sulfate and 2 mmol/L ethylenediamine tetraacetic acid at 65°C for 30 minutes. Filters were air dried and exposed to X-OMAT AR films (Kodak, Rochester, NY) with intensifying screens. Densitometry on autoradiograms from slot blot assays (5 µg total RNA/slot) was performed using a laser densitometer (LKB2202 Ultrascan, LKB, Piscataway, NJ). The optical density was determined for a 24-hour exposure for VEGF and an exposure of more than 48 hours for α -actin.

Competition of [¹²⁵I]VEGF₁₆₅ binding by brain tumor cyst fluid

Radioiodination of VEGF₁₆₅ was done as described before (36). Binding experiments were performed on fetal bovine aortic endothelial cells as described previously (23). Five to 10% (v/v) of brain tumor cyst fluids and plasma were used for competition experiments, and recombinant VEGF₁₆₅ (18) was used for the calibration curve.

Reverse transcriptase PCR technique

Total RNA was isolated from tumor tissue or cells as described above. The PCR technique was similar to one described before (59). One microgram of total RNA and 50 pmol hexamer primers were annealed in 50 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 10 mmol/L dithiothreitol, 6 mmol/L MgCl₂, and 500 µmol/L deoxynucleoside triphosphates for 10 minutes at room temperature. The hexamer primers were extended at 42°C for 1 hour using 7 U Moloney murine leukemia virus reverse transcriptase (Gibco-Bethesda Research Laboratories, Gaithersburg, MD). After first-strand synthesis, cDNA was amplified using PCR. The reverse transcriptase reaction was added to 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3, 1.5 mmol/L MgCl₂, 0.01% gelatin, 0.05% Tween 20/Nonidet P40, 200 µmol/L deoxynucleotide triphosphates, 100 µmol/L of each specific primer, and 1 U Taq polymerase (Amersham, Buckinghamshire, UK).

The cycling temperatures and timing were: 95°C for 1 minute, 50°C for 2 minutes, and 72°C for 3 minutes. Thirty cycles of amplification were performed. The two primers used for VEGF cDNA amplification were described before (5). Southern blot hybridization for positive identification of the PCR fragments was done as described previously using a VEGF cDNA probe (59).

Preparation of cell supernatant and Western blot analysis

Human glioma cells were seeded in T75 culture flasks and cultivated as described. When the cells reached confluency, they were washed with phosphate-buffered saline (PBS), and

media were replaced by low-serum-containing (2%) media. Samples were collected after 3 days of incubation, followed by centrifugation and filtration. Cyst fluid was collected as described. A prepurification procedure was performed by acidic precipitation (pH 3.5) followed by centrifugation. The small heparin-sepharose columns (0.5 ml) were equilibrated with PBS and 0.4 mol/L NaCl. Then 35 ml conditioned medium (0.4 mol/L NaCl) or 7 ml cyst fluid (0.4 mol/L NaCl) was loaded. The columns were washed with PBS and 0.4 mol/L NaCl. Elution was done with PBS containing 1 mol/L NaCl. For Western blot analysis the heparin-binding proteins were separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis under nonreducing conditions. The proteins of the cyst fluids were concentrated 3-fold by acetone precipitation before loading. Then 25 µl of each sample (fraction with highest protein content) was applied. After gel electrophoresis, electroblotting onto Immobilon-P transfer membranes (Millipore, Bedford, MA) was performed. Nonspecific staining was blocked with bovine serum albumin. The first antibody was a polyclonal rabbit antiserum (1:500) against recombinant VEGF₁₆₅. The second antibody was an antirabbit immunoglobulin G conjugated with alkaline phosphatase (1:5000; Promega, Madison, WI). The detection of immunoreactive proteins was done using nitro blue tetrazolium salt/5-bromo-4-chloro-3-indolyl-phosphate (Promega) as substrates for the antibody bound enzymes.

RESULTS

VEGF/VPF and PIGF mRNA levels in brain tumors

Total RNA from 26 different brain tumor tissue samples was used to evaluate the VEGF/VPF mRNA levels by Northern blot analysis. Two low-grade astrocytomas were weakly positive; one was negative for VEGF/VPF gene expression. The pilocytic astrocytoma was clearly positive (Table 1). Anaplastic astrocytomas and glioblastomas (high-grade tumors) are very heterogeneous for VEGF/VPF expression (Fig. 1A). However, 7 of 10 tumor samples are clearly positive for VEGF/VPF gene expression. It was interesting to note that in two tumor samples from the same patient the expression level was very different and increased markedly from one excisional procedure to the following, separated by an interval of 4 months (Table 1 and Fig. 1A). Meningiomas are found to be tumors with low VEGF/VPF mRNA levels if compared with glioblastomas or anaplastic astrocytomas (Table 1). Both medulloblastomas, however, also had low VEGF/VPF mRNA levels. By using a slot blot assay for quantification we found that the expression level can increase to more than 20- to 50-fold in anaplastic gliomas or glioblastomas if compared with low-grade tumors (not shown).

So far no data were available for PIGF gene expression in tumor tissue samples. Because PIGF can compete with VEGF/VPF for receptor binding, we analyzed the expression level of this gene in a subset of our tumor samples. Most of the tumor samples did not show significant PIGF gene expression. However, 1 anaplastic astrocytoma and 2 meningiomas out of 12 samples (25%) displayed very high PIGF expression levels (Fig. 2). All three tumors were by coincidence from women.

TABLE 1. Expression of Vascular Endothelial Growth Factor/Vascular Permeability Factor, Placenta Growth Factor, and Vascular Endothelial Growth Factor Receptor Genes in Brain Tumors^a

No.	Brain Tumors (Type)	WHO	VEGF Expression	PIGF Expression	VEGF Receptors	
					<i>flt-1</i> Expression	KDR Expression
1	Pilocytic astrocytoma	I	++	ND	(+)	+
2	Astrocytoma	II	(+)	(+)	+	+
3	Astrocytoma	II	(+)	(+)	++	+
4	Astrocytoma	II	-	ND	(+)	(+)
5	Anaplastic astrocytoma	III	+	+++	++	++
6	Anaplastic astrocytoma ^b	III	+	(+)	+	+
7	Anaplastic astrocytoma ^b	III	+++	(+)	+	+++
8	Glioblastoma	IV	(+)	(+)	-	-
9	Glioblastoma ^c	IV	(+)	(+)	+	+
10	Glioblastoma ^c	IV	++	(+)	++	+
11	Glioblastoma	IV	(+)	(+)	(+)	+
12	Glioblastoma	IV	++	ND	ND	ND
13	Glioblastoma	IV	+++	ND	ND	ND
14	Glioblastoma	IV	++	ND	+++	++
15	Anaplastic oligoastrocytoma	III	++	ND	+++	+++
16	Oligodendroglioma	III	(+)	(+)	++	++
17	Medulloblastoma	IV	(+)	ND	(+)	(+)
18	Medulloblastoma	IV	(+)	ND	(+)	(+)
19	Meningioma	I	(+)	+++	++	++
20	Meningioma	I	(+)	+++	++	++
21	Meningioma	I	(+)	ND	(+)	+
22	Meningioma	I	(+)	ND	+++	+++
23	Meningioma	I	(+)	ND	++	++
24	Meningioma	I	+	ND	++	+++
25	Adenocarcinoma		+	ND	-	-
26	Malignant ^d		+++	ND	++	++

^a VEGF, vascular endothelial growth factor; PIGF, placenta growth factor; *flt-1*, *fms*-like tyrosine kinase; KDR, kinase insert domain receptor. Tumor grading is according to the World Health Organization (WHO) classification of brain tumors. Pilocytic astrocytoma, Grade I; low-grade glioma, Grade II; high-grade glioma, Grade III or IV. The expression levels of the growth factor and growth factor receptor genes were estimated from Northern blot and slot blot analysis using tumor RNA from each patient. Quantification: -, negative; (+), slightly positive; +, low positive; ++, positive; +++, highly positive; ND, not determined.

^b No. 6 and 7 were samples from the same patient reoperated 4 months after the first treatment.

^c No. 9 and 10 are samples from the same patient but from different regions of the tumor.

^d No. 26 is an unusual malignant partly glial tumor from a 2.5-month-old infant.

Detection of two VEGF/VPF receptor mRNAs in brain tumor tissues

So far two receptor tyrosine kinases (*flt-1* and KDR types) are identified as receptors for VEGF/VPF that can be found on vascular endothelial cells. Because very little is known about VEGF/VPF receptor gene expression in tumor tissue samples, we analyzed the gene expression level of *flt-1* and KDR in some of our tumor samples (Fig. 3). With the exception of two tumors (samples 8 and 11), all examined samples showed similar expression levels of the *flt-1* genes. No obvious correlation was found with VEGF/VPF mRNA levels. However, in the three cases with elevated PIGF gene expression, both VEGF/VPF receptors are easily detectable. The expression level of the KDR gene was generally lower, indicated by a longer exposure time, and more heterogeneous if compared with *flt-1* (Fig. 4). We were not able to find a strong correlation with VEGF/VPF or

PIGF expression levels and found no obvious correlation between the two receptors for their gene expression. However, these results were based on a low number of samples but clearly indicated that in most of the tumor samples, both VEGF/VPF receptor mRNAs can be found, and that the expression patterns of both genes are identical to the mRNA patterns found in vascular endothelial cells.

Expression of VEGF/VPF and its receptors in brain tumor cell lines and endothelial cells

To analyze the possibility that tumor cell lines express one of the known VEGF/VPF receptors, we used two different brain tumor cell lines and two human endothelial cell types for the analysis of VEGF/VPF, PIGF, and VEGF/VPF receptor gene expression. These two endothelial cell types were selected because human brain microvessel endothelial cells were not

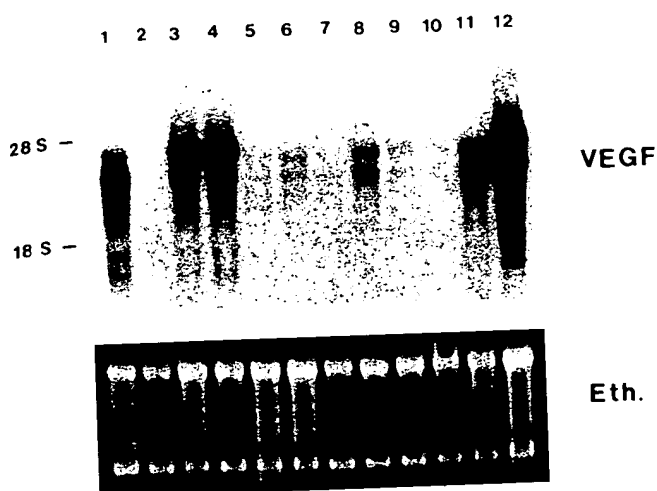


FIGURE 1. Northern blot analysis of VEGF/VPF gene expression in human brain tumors. Five micrograms of total RNA from each tumor were size fractionated on agarose gels, blotted to nitrocellulose membrane filters, and hybridized to human VEGF/VPF cDNA that had been ^{32}P labeled by random priming. Hybridization (2×10^6 cpm/ml) was performed in $2 \times$ standard saline citrate, $10 \times$ Denhardt's solution, 5 mmol/L ethylenediamine tetraacetic acid, 0.1% sodium dodecyl sulfate, and 30 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA at 65°C for 16 hours. The lanes contain total RNA from tumor sample tissues shown in Table 1. Lane 1, SNB-19; Lane 2, astrocytoma 4; Lane 3, oligoastrocytoma 15; Lane 4, glioblastoma 14; Lane 5, meningioma 22; Lane 6, meningioma 23; Lane 7, meningioma 21; Lane 8, meningioma 24; Lane 9, medulloblastoma 18; Lane 10, medulloblastoma 17; Lane 11, adenocarcinoma 25; and Lane 12, malignant brain tumor 26. Sample loading of RNA is indicated by the ethidium bromide (Eth)-stained gel.

available for such studies, and umbilical vein endothelial cells are especially well characterized human vascular endothelial cells. VEGF/VPF is highly expressed in astrocytoma cells as well as in glioblastoma cells in culture (Fig. 5). No expression of the PIGF gene was found in brain tumor cell lines, but a relatively high expression level was found in human vascular endothelial cells from the umbilical cord. The *flt-1* receptor is expressed at a very low rate in the glioblastoma cell line, and a very high expression level is found in umbilical vein endothelial cells, but no expression could be detected in human omental endothelial cells. The second VEGF/VPF receptor encoded by the KDR gene is expressed in both endothelial cell types but not in tumor cells (Fig. 5).

Identification of different VEGF/VPF isoforms by reverse transcriptase PCR

Our Northern blot studies (Fig. 1) did not distinguish between the mRNAs coding for the different VEGF/VPF splice forms. To address this question, first-strand synthesis was done with random primers, and cDNA amplification was performed with primers located at the beginning and at the end of the open reading frame for VEGF/VPF. The products were

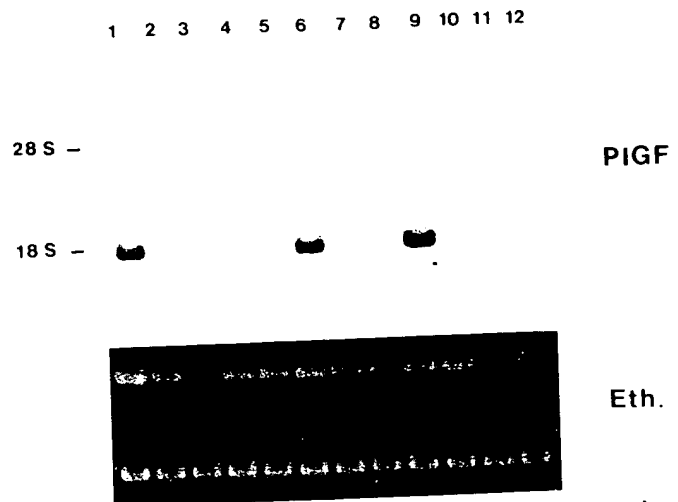


FIGURE 2. Northern blots of brain tumor RNA hybridized to a PIGF probe. Five micrograms of total RNA were hybridized to a human PIGF cDNA that had been ^{32}P labeled by random priming. Hybridization was performed as described in Figure 1. The lanes contain total RNA from tumor tissue samples shown in Table 1. Lane 1, meningioma 20; Lane 2, anaplastic astrocytoma 6; Lane 3, anaplastic astrocytoma 7; Lane 4, glioblastoma 10; Lane 5, glioblastoma 9; Lane 6, meningioma 19; Lane 7, astrocytoma 2; Lane 8, glioblastoma 11; Lane 9, anaplastic astrocytoma 5; Lane 10, oligodendroglioma 16; Lane 11, glioblastoma 8; and Lane 12, astrocytoma 3. Sample loading of RNA is indicated by the ethidium bromide (Eth)-stained gel.

separated by agarose gel electrophoresis (Fig. 6) and confirmed by Southern blotting as described before (58) using ^{32}P -labeled VEGF/VPF cDNA as a probe. Three bands were detected in most of the tumor samples with sizes of about 450 base pairs (bp), 590 bp, and 660 bp. Although our PCR experiments were not done in a semiquantitative way, the 590-bp band was always the most prominent band, followed by the 450- and 660-bp bands. These results may indicate that three different splice forms (VEGF₁₆₅, VEGF₁₂₁, and VEGF₁₈₉) are generated in most of the brain tumor samples. Very similar results were obtained by Berkman et al. (3) using an RNase protection assay and RNA from brain neoplasms.

Identification of VEGF/VPF-like activity in brain tumor cyst fluids

The concentration of VEGF/VPF-like activity in human plasma and in the fluid of a low-grade astrocytoma cyst was low, about 2.9 to 4.5 ng/ml, a concentration range (0.29–0.45 ng/ml of the diluted sample) at the beginning of the linear range of the calibration curve obtained with VEGF₁₆₅ (Table 2). Three glioblastomas showed significant levels of the VEGF/VPF-like activity in their cyst fluids. The elevation is at least sixfold but can increase in some patients over time to more than 45-fold if compared with plasma concentrations. It is interesting to note that in Patient D the concentration of the VEGF/VPF-like activity increased during tumor progression from 44 to 163 ng/ml during an interval of 5 months (Table 2).

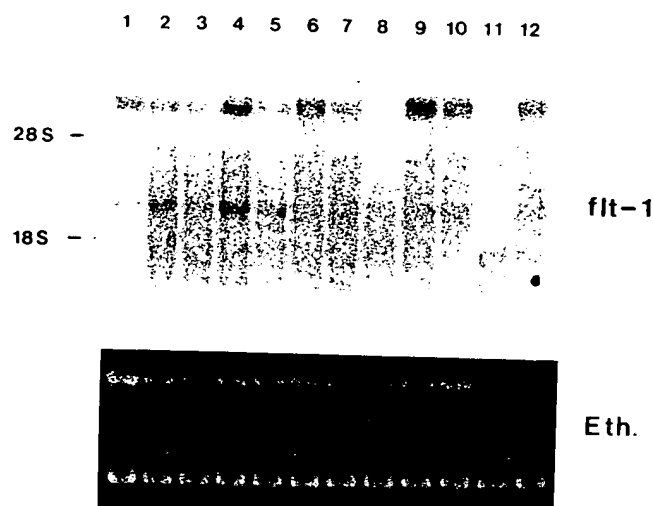


FIGURE 3. Northern blots of brain tumor RNA hybridized to a VEGF/VPF receptor *flt-1* probe. Five micrograms of total RNA were hybridized to a human *flt-1* cDNA that had been ^{32}P labeled by random priming. Hybridization was performed as described in Figure 1. Lanes 1 to 12 contain the same samples as indicated in Figure 2. Sample loading of RNA is indicated by the ethidium bromide (Eth)-stained gel.

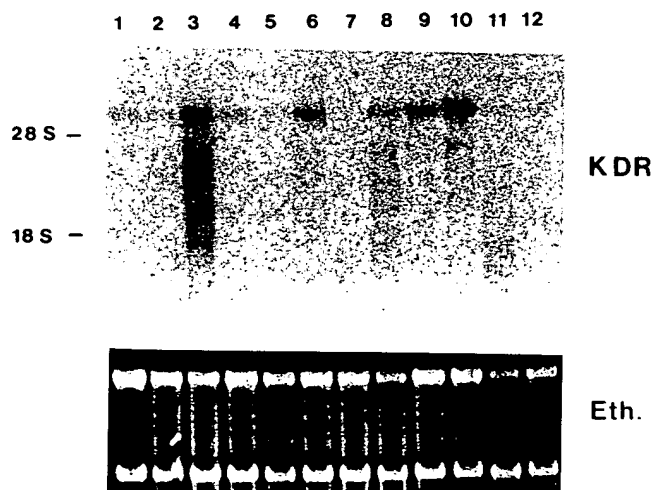


FIGURE 4. Northern blots of brain tumor RNA hybridized to a VEGF/VPF receptor KDR probe. Five micrograms of total RNA were hybridized to a human KDR cDNA that had been ^{32}P labeled by random priming. Hybridization was performed as described in Figure 1. Lanes 1 to 12 contain the same samples as indicated in Figure 2. Sample loading of RNA is indicated by the ethidium bromide (Eth)-stained gel.

Western blot analysis

Western blot analysis and heparin-affinity chromatography was used for the demonstration of the secretion and presence of heparin-binding VEGF/VPF in brain tumor cell lines and cyst fluids (Fig. 7). Both SNB-19 and HGBM-1 cells showed immunoreactive bands with M_r s of approximately 39,000 to 42,000, very similar to that shown for recombinant VEGF₁₆₅. HGBM-1 seems

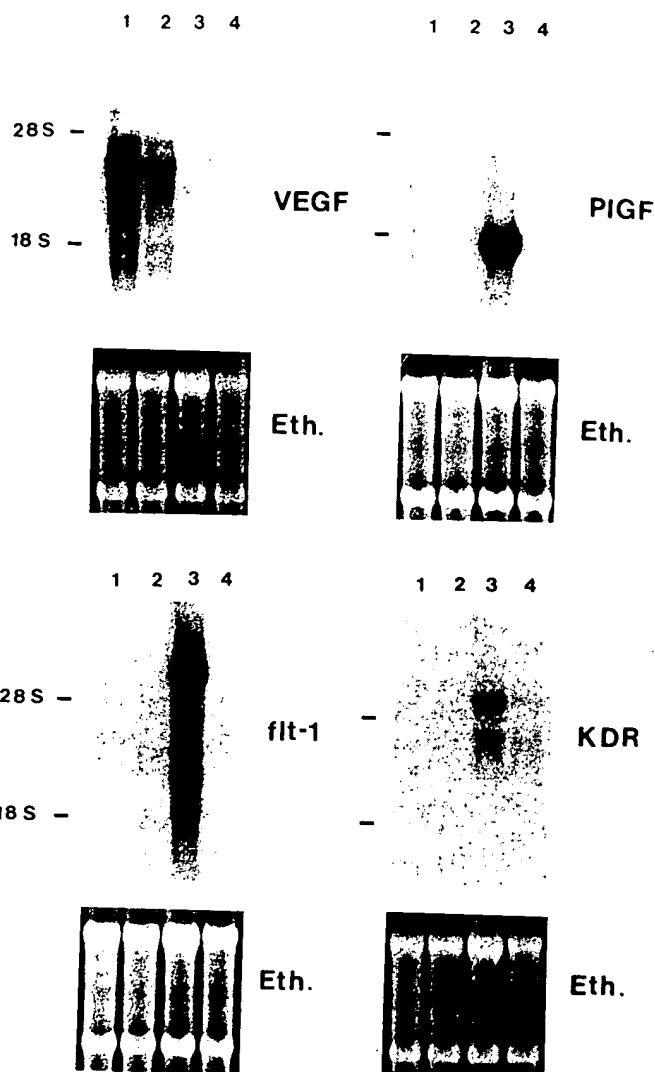


FIGURE 5. Expression of VEGF/VPF, PIGF, and the VEGF/VPF receptors (*flt-1* and KDR) in human brain tumor cell lines and in human endothelial cells. Five micrograms of total RNA were hybridized to the human cDNA probes, which had been ^{32}P labeled by random priming. Hybridization was performed as described in Figure 1. Lane 1, SNB-19 astrocytoma cell line; Lane 2, HGBM-1 cell line; Lane 3, human umbilical vein endothelial cells; and Lane 4, human omental endothelial cells.

to produce both forms, unglycosylated and glycosylated forms ($M_r \approx 46$ kDa) at similar rates. SNB-19 cells as well as the VEGF/VPF protein detectable in cyst fluids seem to be less glycosylated compared with HGBM-1 cells.

DISCUSSION

Primary malignant brain tumors tend to regrow regardless of the treatment administered. Additionally, differentiated low-grade gliomas may during the course of time undergo anaplastic development and become dedifferentiated fast-

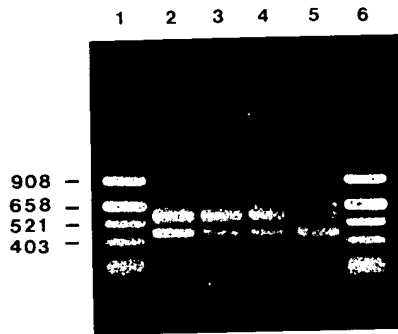


FIGURE 6. Detection of different VEGF/VPF cDNA variants by reverse transcriptase PCR using RNA from brain tumors and SNB-19 cells. Lanes 1 and 6, *AluI*-digested pBR 322 marker; Lane 2, SNB-19 cells; Lane 3, oligoastrocytoma 15; Lane 4, glioblastoma 14; and Lane 5, meningioma 24 (see Table 1).

TABLE 2. Detection of Vascular Endothelial Growth Factor/Vascular Permeability Factor-like Activity in Samples of Cyst Fluids Measured by a Radioreceptor Assay*

Sample	Date	VEGF/VPF-like Concentration (ng/ml)
A-1	April 8, 1991	$\leq 4.5 \pm 0.05$
A-2	April 29, 1991	$\leq 3.0 \pm 0.09$
A-3	May 13, 1991	$\leq 2.9 \pm 0.05$
B-1	Jan 30, 1991	20.5 ± 0.97
C-1	March 26, 1991	37.0 ± 3.60
C-2	April 3, 1991	40.0 ± 0.75
D-1	Oct 4, 1990	44.0 ± 5.23
D-2	Dec 20, 1990	86.0 ± 1.94
D-3	April 8, 1991	163.0 ± 29.63
D-4	May 2, 1991	146.0 ± 12.97
Plasma		$\leq 3.4 \pm 0.29$

* VEGF/VPF, vascular endothelial growth factor/vascular permeability factor. One to four samples of cyst fluid were collected from one patient with low-grade astrocytoma (Sample A) and from three patients with anaplastic astrocytomas (Samples B-D) at different time points as indicated. As reference, sample normal human plasma was used from a local blood bank. FBAE cells were incubated with 1 ng/ml [¹²⁵I]VEGF₁₆₅ in the presence of tumor cyst fluids or control human plasma (10% v/v) for 3 hours. After washing the cell three times, bound radioactivity was measured in a γ -counter. For calibration, cells were incubated with 1 to 50 ng/ml unlabeled recombinant VEGF₁₆₅. The concentrations of the VEGF/VPF-like activity in the samples were calculated from the regression curve obtained from the calibration curve. Data are expressed as the mean \pm SD for plasma and brain cyst fluid.

growing neoplasms. Malignancy is characterized by high cellular density, nuclear and cellular pleomorphism, mitosis, the presence of necrosis, capillary sprouting, and endothelial cell proliferation (7, 14, 45, 46, 61). At the present time the fundamental mechanism that triggers the cascade of events that lead to the progression of an initially low-grade quiescent or slow-growing glioma to a highly proliferative anaplastic tumor with a dense three-dimensional capillary network is not known. Although differentiated low-grade gliomas may reach an im-

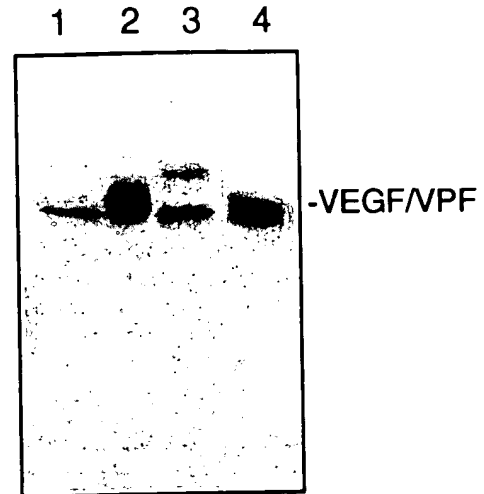


FIGURE 7. Immunoblotting of cell supernatant and cyst fluid protein after concentration by heparin-sepharose from SNB-19 (Lane 1), 50 ng recombinant human VEGF₁₆₅ (Lane 2), HGBM-1 (Lane 3), and cyst fluid from a patient with glioblastoma (Lane 4). A specific reaction with the VEGF/VPF antibody is demonstrated for the unreduced, dimeric protein after a purification and concentration step with heparin-sepharose and acetone precipitation.

pressive volume without showing morphological signs of anaplasia, it seems that a high proliferative rate of tumor cells requiring an increased blood supply is not possible without concomitant acceleration of angiogenesis. High vascularization, i.e., capillary and endothelial cell proliferation and simultaneously increased capillary permeability with the formation of edema, strongly suggest an active role of VEGF/VPF.

Initially, Folkman (20) hypothesized that solid tumors may secrete specific factors (tumor angiogenesis factors) for the proliferation of endothelial cells, which may stimulate the formation of new blood vessels on an expanding tumor volume. In the last 10 to 15 years, directly or indirectly acting angiogenic proteins and their receptors have been identified in central nervous system neoplasms and in brain tumor cyst fluids, including acidic fibroblast growth factor (37, 53, 54) basic fibroblast growth factor (1, 53, 54), insulin-like growth factors 1 and 2 (43), platelet-derived growth factor (34) and epidermal growth factor receptor mRNAs (19, 29, 33), and transforming growth factors α (33) and β (32, 54). The roles of these angiogenic proteins in the biology of primary brain tumors are still not understood. An essential point is that several receptors for these angiogenic molecules also have been found on tumor cells, and not only on endothelial cells. This fact makes it difficult to estimate whether these growth-regulatory molecules are involved in an autocrine and/or paracrine mode of tumor cell stimulation or whether they also stimulate endothelial cells in a paracrine way during tumor progression.

Our previous studies (40) showed independently from others (1, 3) the presence of VEGF/VPF in brain tumors. In the present study we have demonstrated the expression of the VEGF/VPF and the PIGF genes in 26 different brain tumor

tissue specimens. We have found that VEGF/VPF gene expression is up-regulated in many high-grade gliomas, and that some brain tumors also show elevated levels of the PIGF mRNA. From our earlier studies it was not possible to determine which of the VEGF/VPF isoforms are secreted from tumor cells. These data and the results published from Berkman et al. (3) suggest that probably two VEGF/VPF isoforms, VEGF₁₂₁ and VEGF₁₆₅, are secreted from tumor cells. The two known VEGF receptors are detected in brain tumors, but their expression levels showed no obvious correlations with the tumor grades. The two VEGF/VPF receptors are expressed in human vascular endothelial cells but not in brain tumor-derived cell lines, supporting the concept of paracrine growth stimulation, assuming that these receptors are expressed in the brain microvasculature (40). The concentration of VEGF/VPF-like activity was measured in cyst fluids from patients and was elevated up to 45-fold in patients with high-grade gliomas compared with the cyst fluid from low-grade astrocytomas or human plasma. Accordingly, the extent of edema in the vicinity of malignant brain tumors may be interpreted in association with the concentration of VEGF/VPF, which could be assessed quantitatively in the fluid from brain tumor cysts. However, our binding studies could not determine if VEGF/VPF or a similar growth factor protein competes with VEGF₁₆₅ for receptor binding. It is important to consider this limitation, because we showed in earlier studies that PIGF also can compete with VEGF/VPF for receptor binding (23).

Expression of VEGF/VPF cannot be proposed as an explanation for the anaplastic progression of brain gliomas or as the exclusive cause for capillary and endothelial proliferation in brain tumors. Other growth factor receptors such as the platelet-derived growth factor β -type receptor are also up-regulated in the vessels derived from human gliomas and may be involved in capillary formation (39). VEGF/VPF seems rather to represent the key molecule for starting neovascularization and a sensitive measure of the angiogenic potential of a tumor. It is therefore not surprising that pilocytic astrocytomas, which may be rich in capillary blood vessels, express VEGF/VPF. The same reasoning applies to meningiomas, whose abundant vascularization may be visualized angiographically by means of contrast-enhanced computed tomographic scanning and may be a result of surgery. From the present observation one could suppose there is at least a partial role of the VEGF/VPF molecule in the formation of the edema surrounding many meningiomas. Five out of the six meningiomas analyzed in the present study had marked zones of edema resembling those of malignant gliomas or metastases.

From these results, we conclude that an obligatory linear correlation between the level of VEGF/VPF gene expression and the quantity of receptor gene expression should not be expected. Differences between the levels of the factor and those of the receptor also may change in the course of the evolution of the tumor and modify the ratio of both. We also mentioned before that the level of VEGF/VPF expression can be very heterogeneous in our samples. This heterogeneity also can be observed in normal brain samples (5) and may in our case demonstrate the heterogeneity of our collected tumor samples and the influence of the surrounding tissues rather than big

differences in the tumor core. Moreover, we know that apparently selected cell clones in glioblastomas can express the VEGF/VPF gene under certain circumstances (40).

Different growth factors that are simultaneously up-regulated in malignant brain tumors are not likely to coexist without reciprocal influence (21). The knowledge of the essential correlations is indispensable for a better understanding of the biology of primary malignant brain tumors. The stimulus for the up-regulation of growth factors and particularly of VEGF/VPF in malignant brain tumors is not known, even given the fact that hypoxia may be able to act as an activator of the VEGF/VPF gene (40, 51).

VEGF/VPF may represent a useful marker and measurable element of brain tumor biology, allowing for the assessment of vascularity and angiogenic activity, respectively, in different phases of the evolution of brain gliomas. This molecule could be a diagnostic aid, e.g., to solve the problem of grading in cases in which common-type astrocytomas and oligoastrocytomas classified as Grade II behave clinically as Grade III. Thus it could be a parameter for prognosis. Therapeutic implications of the data collected may be envisaged if blocking antibodies to all VEGF/VPF types are available or if receptor antagonists can be developed. For such purposes, thorough studies of the receptors will be necessary.

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COMMENTS

Some 10 years ago, a group of soluble factors was isolated from tumor tissue and found to have strong angiogenic properties (1). Many of these factors we now know as members of the fibroblast growth factor family, but other growth factors such as platelet-derived growth factor and transforming growth factor also seem to play important roles in the complex pathophysiology of tumor angiogenesis. Because capillary endothelial hyperplasia is a well-recognized if not peculiar histopathological manifestation of anaplastic astrocytomas, understandably there is a great deal of interest in determining the key factors that cause capillary endothelial hyperplasia in the hope that the antagonism of these factors may prove to have therapeutic utility as an antiangiogenic strategy. Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) is the most recently characterized of the angiogenic factors, and in this report by Weindel et al., VEGF/VPF seems to be more highly expressed in malignant gliomas than in lower-grade gliomas in a series of 26 tumor specimens. In addition, the authors have demonstrated that glioma cells in culture secrete VEGF/VPF but not the VEGF/VPF receptors, supporting the hypothesis that malignant glioma cells may act in a paracrine fashion on cerebrovascular endothelial cells to cause capillary endothelial hyperplasia. However, the authors have not studied the VEGF/VPF receptor status of the brain microvasculature, which could be performed with an in situ hybridization analysis to validate fully the paracrine hypothesis as stated. I believe this manuscript is an essential addition to the literature on VEGF/VPF and is one of the first to study its expression in human brain tumors.

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1. Shing Y, Folkman J, Sullivan R, Butterfield C, Murray J, Klagsbrun M: Heparin affinity: Purification of a tumor-derived capillary endothelial cell growth factor. *Science* 223:1296-1299, 1984.

The role of VPF in brain tumors has been of significant interest since it was first described. It is fascinating to observe how the roles of growth factors continue to enlarge. VPF or VEGF has multiple functions. It is clearly implicated in angiogenesis, permeability, and the release of von Willebrand factor, as well as thromboplasmin activity.